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Effects of Salinity, Formalin Concentration and Buffer on Quality of Preservation of Southern Flounder (*Paralichthys lethostigma*) Larvae

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Shrinkage, distortion and deterioration of preserved fish larvae can be minimized by judicious choice of fixative/preservative solutions. A seven year factorial experiment was conducted with *Paralichthys lethostigma* larvae fixed and preserved in 12 different solutions comparable to those used in ichthyoplankton collections. The factors were: salinity (0‰, 35‰), formalin (4%, 7%, 10%) and buffer (none, sodium borate). During the experiment, preserved larvae had standard lengths that ranged from 90.6% to 100.7% of live SL. Salt water caused significant shrinkage (to 90.6–94.8% of live SL). Unbuffered 4% formalin in fresh water caused the least change from live length (97.4–100.7% of live SL). Unbuffered and buffered 10% formalin in salt water caused the least change after fixation (92.7–94.8% of live SL). pH ranged from 3.9 to 8.9. Only unbuffered saltwater solutions maintained nearly suitable pH (6.9–8.0), but only for the first year. Unbuffered freshwater formalin preserved pigment the best but apparently decalcified the skeleton. Buffered saltwater formalin preserved the skeleton but bleached pigment. From this experiment and data from more than three years of additional observations with acetate buffered formalin, we conclude that a solution suitable for fixation and preservation of fish larvae is 4% formalin in distilled water buffered with 1% sodium acetate.

THE value of morphometric studies (and sometimes validity of identifications) of fish larvae depends on the accuracy and precision of measurement of body parts and body length. Factors that may affect these measurements, such as prefixation handling and type and strength of preservative, need to be examined to determine if valid comparisons can be made between measurements of fresh and preserved specimens or between fish preserved by different techniques. Larval fish, because they are soft-bodied, are more sensitive to these factors than larger fish. Therefore, whenever the precision of measurements is critical, care should be taken to standardize methods and preservatives so that error associated with shrinkage and distortion is minimal.

Most ichthyoplankton collections are both fixed and preserved in formalin solutions (one exception is the use of ethanol in otolith studies). Commercial formalin is a 39% aqueous solution of the gas formaldehyde. Concentration of the diluted solution is usually chosen to effect a compromise between decomposition by bacteria and autolysis at low concentrations and greater likelihood of demineralization by low pH at high concentrations (with time, formaldehyde in aqueous solution undergoes an addition reaction to yield formic acid). A solution of less than optimum strength may be chosen in an attempt to avoid noxious vapors while processing specimens. Solutions commonly are buffered with borax (sodium borate) or limestone (calcium carbonate) (Smith and Richardson, 1977; Taylor, 1977; Ahlstrom, 1976).

The likelihood and degree of shrinkage or

distortion of preserved fish larvae are influenced by a number of factors: specimen size (Hay, 1982); skeletal rigidity, tissue damage, time between death and fixation (Theilacker, 1980); formalin concentration (Hay, 1982; Blaxter 1971); solution osmolality, which depends partly on salinity (Hay, 1982; Blaxter, 1971) and time in the solution (Hay, 1982; Theilacker, 1980; Blaxter, 1971). Osmotic strength of the solution, which usually depends on the amount of salt, formalin and buffer, is an important factor often overlooked. For most sampling, fixative solutions are prepared with water from the collection site. Salinity could vary from site to site in a series of collections, especially in estuaries. During sorting, specimens often are transferred to fresh solutions, which sometimes differ drastically in osmolality.

Another concern is that of structural or pigment deterioration induced by extremes in pH of the preservative solution. Calcium carbonate will dissolve at a pH below 8.2 (Griffiths et al., 1976). Demineralization and reduced staining with alizarin red S has been noted in larger fish kept in unbuffered formalin solutions (pH below 7.0, Taylor, 1977), and, in larvae, demineralization can be severe enough to cause skeletal disintegration. At low pH, contraction of proteins may cause specimens to become brittle and break easily (Steedman, 1976a). At high pH (near 8), swelling of proteins may cause specimens to appear translucent, because of reduced density (Steedman, 1976a). This process may result in a cleared specimen, as often occurs in borax-buffered formalin (Taylor, 1977), and can result in soft tissue disintegration. Proteins are least soluble and most stable at their isoelectric points, which are mostly below pH 7 (Steedman, 1976a), but a slightly higher pH increases flexibility (Steedman 1976a) and decreases fragility. Most pigments in marine zooplankton, including fish larvae, become bleached or dissolved after one to two years at a pH of 8 or above, probably as a result of protein breakdown (Steedman, 1976a). For minimizing demineralization, brittleness, bleaching, and clearing, the best compromise in pH appears to be in the range 7.0–7.5.

In this paper we examine the relative effects of 12 solutions comparable to those commonly used to fix and preserve fish larvae captured in fresh or salt water. We describe changes in standard length (SL) and condition of southern flounder (*Paralichthys lethostigma*) larvae and changes in pH of the preservative during a seven year factorial experiment. The longest larval shrinkage experiments previously reported lasted for only 1.5 years (Schnack and Rosenthal, 1978). There are no published records of pH changes over time in larval fish preservative solutions.

Materials and Methods

Transforming southern flounder larvae (9.49–13.05 mm SL) were captured in the live box of a stationary plankton net in Walden Creek, a tributary of the Cape Fear River near Southport, North Carolina. Salinity was 5‰. Specimens were kept alive until they were fixed. Seventy-two fish were divided into groups of six replicates and tested over 12 treatment combinations.

Effects of three factors (salinity, formalin concentration, buffer) were investigated. Two water types (0‰ well water, 35‰ artificial seawater), three formalin concentrations (4%, 7%, 10%), and two buffer states (unbuffered, buffered with 1 g sodium borate/300 ml) were utilized in a factorial design. For each of the 12 treatments, six liquid scintillation vials were partially filled with 10 ml of preservative (specimens <2% of volume). Partial filling reserved space for a pH probe. Initial temperature was 24 C. The standard length of each (live) specimen was measured on the left side with an ocular micrometer in a stereomicroscope. Immediately after being measured, each larva was placed in a separate vial, which was firmly capped and stored in the dark at room temperature (ca. 18–28 C). Larvae were remeasured at the following times after fixation: 1 h, 2 h, 4 h, 24 h, 48 h, 72 h, 7 d, 14 d, 4 wk, 8 wk, 1 yr, 6 yr. Evaporation was negligible through the first year, and all measurements were obtained, but after 6 yr, evaporation had occurred in some vials, and the specimens in them were not remeasured, as noted in the results.

The preservative solution pH was monitored in half of the experimental containers (three vials per treatment). In addition, pH was measured in a control set of 12 vials, each containing 10 ml of preservative, but no fish. Determinations were made at 6 h (fish only), 16 h (control only), 24 h (both from here on), 48 h, 72 h, 7 d, 14 d, 21 d, 4 wk, 6 wk, 10 wk, 20 wk, 32 wk, 1 yr, 6 yr. To compare pH changes in the well-water and artificial seawater solutions with those in naturally occurring waters, additional solutions were prepared with 10‰ water from Wal-

TABLE 1. ANALYSIS OF VARIANCE (REPEATED MEASURES DESIGN) OF PERCENT SHRINKAGE DATA. For each of 12 combinations of salinity, formalin, and buffer, six southern flounder larvae stored in individual vials were measured 11 times (1 h-1 yr) after fixation.

Source of variation	df	MS	F
Between subjects	71		
Salinity (S)	1	0.34852	47.7***
Formalin (F)	2	0.00284	0.4
Buffer (B)	1	0.00565	0.8
S × F	2	0.00614	0.8
S × B	1	0.03438	4.7*
F × B	2	0.00041	0.1
S × F × B	2	0.01022	1.4
Subj. within groups (error between)	60	0.00731	
Within subjects	720		
Time (T)	10	0.00346	71.8***
T × S	10	0.00064	13.3***
T × F	20	0.00007	1.4
T × B	10	0.00005	1.1
T × S × F	20	0.00002	0.4
T × S × B	10	0.00002	0.5
T × F × B	20	0.00004	0.8
T × S × F × B	20	0.00002	0.5
T × subj. within groups (error within)	600	0.00005	

* $P < 0.05$.
*** $P < 0.001$.

den Creek and 26‰ water from the Atlantic Ocean off Oak Island, North Carolina. Unbuffered and buffered solutions of 4, 7 and 10% formalin were prepared with water from those two sources and placed in vials; pH was measured at the following times: 1 h, 8 h, 16 h, 24 h, 7 d, 14 d, 21 d, 28 d, 6 wk, 10 wk, 20 wk, 32 wk, 6 yr. Also, pH and osmolality of fresh and old solutions of formalin buffered with sodium acetate were measured.

Because measurements of standard length and pH were repeated on the same fish and solutions, observations made on each replicate over time could not be considered independent. Because independence is a primary assumption in a classical, fixed effects, four-way analysis of variance (salinity × formalin × buffer × time), we used a repeated measures model (Winer, 1971). In our case, both grouping (salinity, formalin, buffer) and trial (repeated measures over time) were fixed effects factors. In this design, each subject (fish larva) is observed at one level of each grouping variable, but at all levels of the trial variable. Briefly, the repeated measures model separates the conventional within-cell variance into variance among subjects within treatment and variance within subjects over trials. The analysis of variance is presented in two parts. The first analyzes main effects and interactions of the three grouping factors. The second analyzes the trial factor and its interactions with grouping factors. All lengths were expressed as a percentage of the initial, unpreserved measurement. Because we could not reject the hypothesis of non-homogeneity of variance (F_{max} test, Sokal and Rohlf, 1981), no transformation was attempted prior to analysis.

To assess structural deterioration after seven years, one specimen from each 7% treatment was x-rayed (Miller and Tucker, 1979) and then cleared and stained with alcian blue for cartilage and alizarin red S for bone (Dingerkus and Uhler, 1977). Alcian blue stains acid mucopolysaccharides of chondromucin (cartilage) and alizarin red S stains calcium in the hydroxyapatite of bone (Pearse, 1968, 1972).

RESULTS

Shrinkage.—On the average, specimens preserved in salt water shrank significantly more

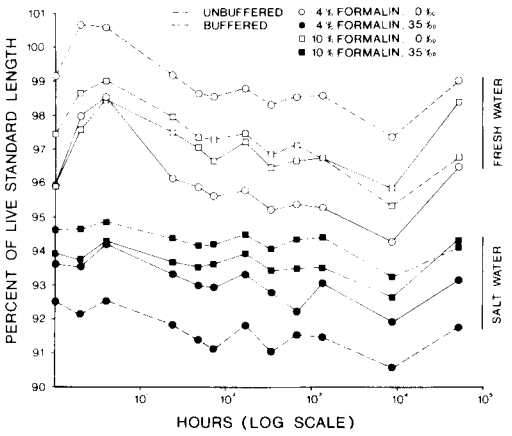

Fig. 1. Mean length changes over time (1 h–6 yr) of southern flounder larvae preserved in eight different solutions.

than those preserved in fresh water (Table 1; Fig. 1). Formalin concentration and buffering had no significant effect on shrinkage. However, there was a significant salinity-buffer interaction whereby larvae in buffered fresh water shrank more than in unbuffered fresh water. This difference seemed to lessen with time, especially with the 10% unbuffered formalin solution and especially beyond 6 wk (1,000 h, Fig. 1). There were significant differences over time (trial factor), but these were confounded with a significant time-salinity interaction. In other words, the effect of time differed, depending on whether fresh or salt water was used. We interpret this as the result of an increase in length in the first 4 h following initial shrinkage in fresh water (Fig. 1). No comparable increase was found with salt water. Between 1 yr and 6 yr, every treatment experienced an increase in length.

pH.—The effect of buffer on pH depended on salinity, as indicated by a highly significant salinity-buffer interaction (Table 2; Fig. 2). pH was somewhat lower in buffered salt water than in buffered fresh water, but pH of unbuffered fresh water was much lower than that of unbuffered salt water. There was also a lesser, but significant interaction of formalin with buffer.

TABLE 2. ANALYSIS OF VARIANCE (REPEATED MEASURES DESIGN) OF pH DATA. For each of 12 combinations of salinity, formalin, and buffer, pH of the fluid in three vials, each containing a single southern flounder larva, was measured 13 times (6 h-1 yr) after fixation.

Source of variation	df	MS	F
Between subjects	35		
Salinity (S)	1	98.75299	320.9***
Formalin (F)	2	4.50128	14.6***
Buffer (B)	1	543.93293	1,767.3***
S × F	2	0.97068	3.2
S × B	1	193.12908	627.5***
F × B	2	1.73970	5.6**
S × F × B	2	0.80897	2.6
Subj. within groups (error between)	24	0.30777	
Within subjects	432		
Time (T)	12	0.46321	41.7***
T × S	12	0.27676	24.9***
T × F	24	0.01992	1.8*
T × B	12	0.40406	36.4***
T × S × F	24	0.01676	1.5
T × S × B	12	0.25337	22.8***
T × F × B	24	0.01357	1.2
T × S × F × B	24	0.02087	1.9**
T × Subj. within groups (error within)	288	0.01111	

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

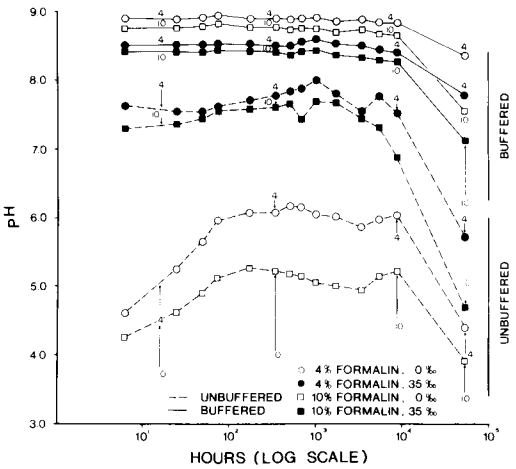

Fig. 2. Mean pH over time (6 h–6 yr) of eight different preservative solutions containing southern flounder larvae. For comparison, numbers (4 and 10) indicate pH in control vials (no fish).

In buffered solutions, formalin concentration seems to have had little effect on pH; in unbuffered solutions, higher formalin yielded lower pH. There were significant differences with time, but those were highly dependent on the particular salinity-formalin-buffer treatments. In general, borax buffered solutions (fresh and salt) maintained the highest pH. The lowest pH occurred in unbuffered fresh water. In all but the unbuffered fresh water, the general trend was for pH to remain fairly constant up to 1 yr, but to drop by 6 yr. This drop was most precipitous in unbuffered saltwater solutions. Only in unbuffered fresh water did the presence of specimens have much influence on pH (Fig. 2). Solutions prepared from the two natural brackish water sources had pH's intermediate between 0‰ and 35‰ water, but these were closer to those of 35‰ water and showed the same trends over time.

Structural and pigment condition.—Although the tips of some fin rays were slightly eroded, specimens in all four salinity-buffer combinations were intact after 7 yr; however, skeletal and pigment differences were striking (Table 3). (In this paragraph, treatments not mentioned were intermediate.) The buffered saltwater specimen formed a sharp, well-defined radiographic image; skeleton and fin rays were relatively dense, radiographically. The skeleton and fin rays of the unbuffered saltwater specimen were visible but faint. None of the skeleton or fin rays of the unbuffered freshwater specimen were distinguishable. Eye, cranial, gut, and marginal and lateral trunk pigmentation was visible in the unbuffered freshwater specimen. Eyes of the buffered saltwater specimen were completely bleached, but traces of cranial and gut pigment were retained. No pigment was visible in the buffered freshwater specimen. The buffered freshwater specimen was already cleared without trypsin digestion; the buffered saltwater specimen was nearly cleared without digestion. Unbuffered freshwater and unbuffered saltwater specimens were resistant to clearing and required 10 d of digestion in three changes of trypsin. The entire skeleton of the unbuffered saltwater specimen, including neural spines, hemal spines, and fin rays, was stained brilliant blue. The entire skeleton of the buffered saltwater specimen, except for parts stained red, was stained a less intense blue. In the buffered saltwater specimen, most bones of the head, all centra, all neural and hemal spines and the

TABLE 3. STRUCTURAL CONDITION OF FOUR SOUTHERN FLOUNDER LARVAE AFTER 7 YR OF PRESERVATION, ASSESSED BY RADIOGRAPHY AND CLEARING AND STAINING.

	Treatment			
	Unbuffered fresh water	Unbuffered salt water	Buffered salt water	Buffered fresh water
Standard length (mm)	9.8	10.9	9.7	9.4
pH at six years	4.1	5.2	7.6*	7.8
Radiographic definition (rank)	4	3	1*	2
Pigment retention (rank)	1*	2	3	4
Time to clear in trypsin (d)	10	10	1	0
Alcian blue staining (rank)	2	1*	4	3
Alizarin red S staining (rank)	4	3	1*	2

* Best treatment by individual criterion.

proximal halves of all caudal fin rays were stained brilliant red. In the unbuffered saltwater specimen, the preopercle, supracleithrum and all but the last 15 neural and hemal arches were lightly stained red. No alizarin was visible in any part of the unbuffered freshwater specimen.

Discussion

Shrinkage.—Degree of shrinkage depends primarily on the osmotic strength of the solution, but also on formalin concentration (Fig. 1). The most shrinkage occurred in unbuffered 4% formalin in salt water, the least in unbuffered 4% formalin in fresh water. In salt water, higher formalin reduced the amount of shrinkage. In fresh water, borax increased the osmotic strength of the solutions, thus increasing shrinkage. Although saltwater solutions caused greater shrinkage, the amplitudes of fluctuations were less than in fresh water (especially in 10% unbuffered and buffered solutions). Of the tested solutions, unbuffered 4% formalin in fresh water would permit the most accurate length measurements; unbuffered or buffered 10% formalin in salt water would permit the most precise measurements over time.

pH.—For the first year, pH of unbuffered saltwater solutions was good—remaining neutral to slightly alkaline (Fig. 2); however, as in the other solutions, there was a decrease between 1 yr and 6 yr. The pH of all buffered solutions was too high, and that of unbuffered freshwater solutions was too low. Of all solutions used in the experiment, only unbuffered saltwater solutions would be nearly appropriate in pH for preservation of fish, but they would require either complete replacement or addition of buffer after 1 yr. Borax raised the pH of both freshwater and saltwater solutions but raised freshwater pH more. Buffering also ameliorated pH fluctuations over time, but did not prevent a decrease between 1 yr and 6 yr (apparently because of lowered buffering capacity). This decrease, for all treatments, probably occurred because the vials were closed continuously for 5 yr and acidic gases could not escape.

Structural and pigment condition.—pH apparently affected condition of the specimens' skeletons and pigment (Table 3). Unbuffered freshwater formalin was the best solution for retaining pigment, but it appeared to decalcify the skeleton, leaving only cartilage and decalcified bone tissue. Decalcification apparently occurred in unbuffered freshwater and saltwater solutions (pH below 6) but not in buffered solutions (pH mostly above 6). Buffered saltwater formalin preserved the skeleton very well but bleached pigment. Both buffered solutions cleared specimens but did not disintegrate them. Specimens did not clear at low pH (unbuffered freshwater and saltwater) and, possibly because of residual acids or because of thorough fixation, resisted clearing in trypsin. Because clearing did occur at pH 7.6, a pH closer to neutral would be advisable.

Recommendations

Some precautions in choice of preservative solutions will maintain the quality of specimens. If sea water is used, little or no buffer will be required; the salinity should be isosmotic with fish fluids (near 15‰). It might be best to start with salinity a little lower than 15‰ to allow for evaporation. Sodium borate should not be used as a buffer, because clearing and disintegration are likely at high pH. Calcium carbonate (limestone) should be used only with caution, because crystals may be deposited on specimens. Hexamine should not be used because it can damage specimens (Steedman, 1976a). Markle (1984) has suggested the use of a sodium phosphate buffering system for general preservation of ichthyoplankton; however, phosphates cannot be used in sea water because of precipitation problems (Steedman, 1976a). Markle also reported precipitation on specimens when phosphate concentrations of 0.019 M or higher were used in distilled water; precipitation did not occur at lower concentrations. Soaking specimens in water reverses fixation and should be avoided; instead ventilation should be relied upon to eliminate formaldehyde fumes (Taylor, 1977). Special solutions and techniques may be necessary for certain types of larvae (Steedman, 1976b).

In this study, except for the effects of high pH (bleaching and clearing), buffered freshwater formalin produced good results. If pH could be maintained at 7.0–7.5 by suitable buffering, freshwater solutions are probably preferable to saltwater solutions. From more than three years' experience with sodium acetate buffered solutions, we conclude that one of the best long term preservatives is 4% formalin in distilled water buffered with sodium acetate. Steedman (1976a) discussed the high stability of seawater solutions buffered with sodium acetate. We measured the pH in ten vials contain-

ing 5–10% by volume of fish larva in a commercial preparation of formalin diluted to 50% with sea water. The resulting solution contained 5% formalin, 1% sodium acetate, 0.75% methanol and 15‰ sea salts. After 19 months, pH ranged 7.3–8.1 and averaged 7.7 (higher than expected in distilled water). No clearing, precipitation or pigment loss have been observed in these or in similarly preserved specimens. We also measured osmolality and pH of a freshly mixed distilled water solution containing 4% formalin, 0.8% sodium acetate, and 0.6% methanol. Osmotic pressure was 384 ± 1 mOsm/kg and pH was 7.0–7.1. The osmolality of teleost blood serum averages close to 350 mOsm/kg (Black, 1957); for adult southern flounders living in full sea water, the value is 348 mOsm/kg (Lasserre and Gilles, 1971). The 4% formalin acetate solution probably would cause little shrinkage or distortion of larvae from osmotic pressure. Balachandran (1976) reported that in a variety of zooplankters, including fish larvae, pigment was retained better in the presence of sodium acetate than with borax, hexamine, calcium carbonate, or sodium bicarbonate. Under the criteria of minimizing shrinkage and distortion, retaining pigment and maintaining suitable pH, 4% formalin buffered with 1% sodium acetate in distilled water has some of the best characteristics of preservatives that are commonly available. It is isosmotic and will maintain pH near neutral; sodium acetate is more soluble than calcium carbonate or sodium phosphate and can be used in salt water or distilled water with less likelihood of precipitates forming. We recommend its use for fish larvae. Phosphate buffering (Markle, 1984) may be just as good or better; however, a comparative study would be necessary to determine this. Because of the known characteristics discussed above, we prefer sodium acetate. Because of possible buffer depletion, annual or biennial pH monitoring is advisable with any solution.

One final point should be emphasized. Generalized factors for correcting preserved lengths to live lengths cannot be extrapolated to different species or different stages (Hay, 1982). Variability in degree of shrinkage renders such extrapolation meaningless.

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